In claim 33, the phrase "increased resistance" is a relative term and requires a comparative basis. All subsequent recitations of "increased resistance" are also rejected

In claim 34, the phrase "increased biomass" is a relative term and requires a comparative basis. All subsequent recitations of "increased biomass" are also rejected.

In claim 40, Applicant is further limiting the plant of claim 28 by stating a "plant" which is selected from a group of names that are not plants, but rather are terms describing groups of plants (e.g. fruit bearing plants, or flowering plants etc). All subsequent recitations in which Applicant defines a plant using the terms or a term as recited in claim 40 are also rejected.

In claims 40 and 70, the word "plant" needs to follow the word "tomato".

In claim 45, the terms "age-related" and "environmental stress-related" are not defined and are unclear. Do these terms need to be in the claim?

In claim 50, it is unclear what is being retained in a product that is "derived" from a cell. If Applicant is claiming progeny that comprise the nucleic acid sequence that was transformed into a cell, then explicitly stating as such, will obviate the rejection. All subsequent recitations in which the term "derived" is recited are also rejected.

In claim 55, the metes and bounds of "aging-induced" cannot be determined since Applicant has not adequately defined this term. All subsequent recitations of "aging-induced" are also rejected.

In claim 56 the preample is not in agreement with the method steps. The preample states a method of inhibiting seed aging and the last step of the method is drawn to "growing said plant" wherein the antisense nucleotide is transcribed. If a plant is present, then there is no longer a seed.

It is unclear why claim 58 is "further comprising" the method of claim 57, when claim 57 is drawn to non-elected material.

It is unclear why claim 66 is "further comprising" the vector of claim 59, when claim 59 is drawn to a vector comprising non-elected material.

In claim 67, the metes and bounds of "physiological disease" have not been defined. How does physiological disease differ from non-physiological disease? All subsequent recitations of "physiological disease" are also rejected.

In claims 28, 45, 51, 56, 67, and 69 part (v) can be deleted because parts (i) and (ii) are directed to non-elected material.

The claims are replete with vague and indefinite terms. A complete review of the claims is required.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Claims 12-17, 24-29, 32-42, 45-46, 48-53, 55-56, 58, and 66-70 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The claims are drawn to a vector, an oligonucleotide, or polynucleotide comprising an antisense nucleotide corresponding to a DNA molecule encoding a senescence-induced elF-5A

wherein said DNA molecule hybridizes under low stringency to SEQ ID NO:11, a vector comprising an antisense nucleotide sequence substantially complementary to a corresponding portion of an RNA sequence encoded by the DNA molecule encoding a senescence-induced elF-5A, wherein the oligonucleotide or polynucleotide comprise about six to about 100 nucleotides, the 5'-non-coding region of the RNA transcript, or comprise the 3'-end of the RNA transcript. The Applicants also claim a method for inhibiting the expression of the elF-5A encoding polynucleotide in a plant wherein said inhibition results in altered senescence of the plant, increased resistance to stress, increased biomass of the plant, delayed fruit softening and spoilage, and increased seed yield comprising transforming a plant with said vector. In addition, Applicants also claim methods of altering age-related and environmental stress-related senescence, method of inhibiting seed aging and increasing resistance to physiological disease in a plant comprising transforming a plant with said vector.

The claimed invention is not supported by an enabling disclosure taking into account the *In re Wands* factors (858F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). *In re Wands* lists a number of factors for determining whether or not undue experimentation would be required by one skilled in the art to make and/or use the invention. These factors are: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples of the invention, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claim.

Applicants isolated a full-length cDNA clone of the senescence-induced elF-5A encoding polynucleotide from a cDNA library constructed from tomato leaves. Applicants do not provide

guidance or examples using any antisense construct complemetary to their cDNA clone for inhibiting expression of the elF-5A protein which Applicants purport will ultimately inhibit senescence. Applicants' claims are drawn to an antisense nucleotide sequence that is substantially complementary to a DNA molecule that will hybridize under low stringency with SEQ ID NO:11. The state of the art teaches isolating DNA fragments using stringent hybridization conditions, does not always select for DNA fragments whose contiguous nucleotide sequence is the same or nearly the same as the probe. Fourgoux-Nicol et al (1999, Plant Molecular Biology 40 :857-872) teach the isolation of a 674bp fragment using a 497bp probe incorporating stringent hybridization conditions comprising three consecutive 30 minute rinses in 2X, 1X and 0.1X SSC with 0.1% SDS at 65^0C (page 859, left column, 2^{nd} paragraph). Fourgoux-Nicol et al also teach that the probe and isolated DNA fragment exhibited a number of sequence differences comprising a 99bp insertion within the probe and a single nucleotide gap, while the DNA fragment contained 2 single nucleotide gaps and together the fragments contained 27 nucleotide mismatches. Taking into account the insertions, gaps and mismatches, the longest stretch of contiguous nucleotides to which the probe could hybridize consisted of 93bp of DNA (page 862, Figure 2). In the present example, the isolated fragment exhibits less than 50% sequence identity with the probe. The selected sequences will encode proteins having modifications including additions, deletions, and substitutions of many amino acids when compared to a protein encoded by SEQ ID NO:11. Therefore, it is unpredictable as to whether any of the antisense molecules will hybridize in vivo to any mRNAs encoding an elF-5A protein and thus inhibit senescence. In addition, in claim 15 Applicant claims an antisense molecule about 6 nucleotides long, as well as an antisense molecule about 100 nucleotides long. Applicant

has not shown that a molecule comprising 6 nucleotides has antisense function and act predictably to suppress elF-5A expression. Lastly, absent any specific guidance as to which regions of the elF-5A encoding polynucleotides can be modified and which regions must not be modified, including the 5' and 3' regions of the RNA transcript, so as to generate an antisense molecule that will hybridize to an endogenous mRNA encoding an elF-5A, one skilled in the art would not be able to use the claimed invention to generate plants whose senescence is inhibited, without undue experimentation.

Applicants purport that inhibiting the synthesis of the elF-5A protein will inhibit senescence. But, Applicants have not reduced to practice their invention. Senescence is a complex, highly regulated process involving multiple proteins in multiple pathways. How senescence occurs is not fully understood to date. It is highly unlikely that one protein controls the senescence process. More likely, there are multiple proteins with redundant functions that are involved in the process to ensure senescence occurs at the appropriate time and place. In addition, if senescence was controlled by a single protein, then mutagenesis experiments should have already uncovered the gene responsible for encoding this protein. But to date, no such gene has been uncovered by chemical or insertional mutageneses. Applicant has not shown that elF-5A alone can regulate or control senescence by itself. It is unpredictable what other proteins are required. Given the lack of guidance and the unpredictability of what other proteins are required, excessive experimentation would be required to make and use the claimed invention.

Therefore, given the breath of the claims; the lack of guidance and examples; the unpredictability; and the state of the art as discussed above, undue experimentation would be required by one skilled in the art to obtain an antisense molecule whose corresponding encoding

DNA molecule would hybridize to SEQ ID NO:11 and when expressed in plants would inhibit just senescence and not disrupt the normal processes required for plant growth.

8. Claims 12-17, 24-29, 32-42, 45-46, 48-53, 55-56, 58, and 66-70 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. This is a written description rejection.

The claims are drawn to a vector, an oligonucleotide, or polynucleotide comprising an antisense nucleotide sequence substantially complementary to a corresponding DNA molecule encoding a senescence-induced elF-5A wherein said DNA molecule hybridizes under low stringency to SEQ ID NO:11, a vector comprising an antisense nucleotide sequence substantially complementary to a corresponding portion of an RNA sequence encoded by the DNA molecule encoding a senescence-induced elF-5A, wherein the oligonucleotide or polynucleotide comprise about six to about 100 nucleotides, and wherein the vector, oligonucleotide or polynucleotide comprise the 5'-non-coding region of the RNA transcript, or comprise the 3'-end of the RNA transcript. The Applicants also claim methods that comprise the above vectors, oligonucleotide, or polynucleotide.

Sequences that hybridize under low stringency with SEQ ID NO:11 or are substantially complementary to a corresponding DNA molecule encoding a senescence-induced elF-5A encompass naturally occurring allelic variants, mutants of elF-5A, as well as sequences encoding proteins having no known elF-5A activity, of which Applicant is not in possession. Absent of such disclosure, one skilled in the art cannot determine the genus of sequences based upon the

disclosure of the cDNA sequence of SEQ ID NO:11 with any certainty or predictability. Accordingly, the specification fails to provide an adequate written description to support the hybridization language or substantially complementary language as set forth in the claims. (See Written Description guidelines published in Federal Register/Vol. 66, No.4/Friday, January 5, 2001/Notices: p.1099-1111).

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

9. Claims 26, 27, 50-53, and 69-70 are rejected under 35 U.S.C. 101 because the claimed invention is directed to non-statutory subject matter.

Claims 26, 27, 50-53, and 69-70 are drawn to a progeny of the transformed plant. Due to Mendelian inheritance of genes, a single gene introduced into a parent plant would only be transferred at most to half the male gametes and half the female gametes. This translates into only two thirds of the progeny having at least a single copy of the transgene and one quarter of the progeny would not carry a copy of the transgene. If the transformed plant is outcrossed, than only one half the progeny will have the transgene. Given that there is no indication that there would be any other distinguishable characteristics of the claimed progeny, it is unclear whether the claimed progeny would be distinguishable from progeny that would occur in nature. The amendment of the claims to recite that the progeny comprise the construct that was introduced into the parent plant would overcome the rejection.

10. Claim 56 is rejected under 35 U.S.C. 101 because the claimed invention is directed to non-statutory subject matter.

The claim is drawn to a method of inhibiting seed aging. Aging cannot be inhibited, because it happens as one day passes to the next, the seed invariably becomes a day older. Just as a perpetual-motion machine is unpatentable, so is a method for inhibiting aging.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

11. Claims 26, 27, 50-53, and 69-70 are rejected under 35 U.S.C. 102(b) as being anticipated by Morrison et al (June, 1998 U.S. Patent 5,763,742)

The claims are drawn to progeny of the transformed plant which because of Mendelian inheritance of genes, a certain proportion of the progeny will not carry the transgene and as such, will not have any distinguishing characteristics. The claims thus read on any progeny from a cross.

Morrison et al disclose a hybrid tomato plant that would be the progeny of a cross between parental lines, and as such, Morrison et al anticipate the claimed invention.

Claims 12-17, 24-27, 48-53, 66, and 69-70 are rejected under 35 U.S.C. 102(b) as being anticipated by de Silva et al (June 1998, U.S. Patent Number 5,767,364)

The claims are drawn to a nucleotide sequence that is substantially complementary to a portion of a corresponding DNA molecule encoding an elF-5A, wherein the nucleotide sequence is in antisense orientation. The claims also encompass a plasmid and vector comprising said nucleotide sequence and a bacterial cell, host cell and plant transformed with said nucleotide sequence.

de Silva et al teach a plant transformed with a nucleotide sequence in antisense orientation. Given the indefiniteness of "substantially complementary" as recited in the present application, the nucleotide sequence of of de Silva et al encompass the claimed material. For purposes of molecular biology, the nucleotide sequence of de Silva et al would be in a plasmid vector and host cell and as such anticipate the claimed invention.

12. No claims are allowed.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Stuart Baum whose telephone number is (703) 305-6997. The examiner can normally be reached on Monday-Friday 8:30AM – 5:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Amy Nelson can be reached on (703) 306-3218. The fax phone numbers for the organization where this application or proceeding is assigned are (703) 305-3014 or (703) 305-3014 for regular communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the legal analyst, Sonya Williams, whose telephone number is (703) 305-2272.

Stuart Baum Ph.D.

November 1, 2002

Phuong Bui 11/4/02
PHUONG T. BUI
PRIMARY EXAMINER

A DOCPHOENIX

BACKFILE DOCUMENT INDEX SHEET

APPL PARTS

_____ IMIS _____
Internal Misc. Paper

_____ LET. _____
Misc. Incoming Letter

_____ 371P _____
PCT Papers in a 371Application

_____ A... _____
Amendment Including Elections

_____ ABST _____
Abstract

_____ ADS _____
Application Data Sheet

_____ AF/D _____
Affidavit or Exhibit Received

_____ APPENDIX _____
Appendix

_____ ARTIFACT _____
Artifact

_____ BIB _____
Bib Data Sheet

_____ CLM _____
Claim

_____ COMPUTER _____
Computer Program Listing

_____ CRFL _____
All CRF Papers for Backfile

_____ DIST _____
Terminal Disclaimer Filed

_____ DRW _____
Drawings

_____ FOR _____
Foreign Reference

_____ FRPR _____
Foreign Priority Papers

_____ IDS _____
IDS Including 1449

_____ NPL _____
Non-Patent Literature

_____ OATH _____
Oath or Declaration

_____ PET. _____
Petition

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Mail Returned by USPS

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Sequence Listing

_____ SPEC _____
Specification

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Specification Not in English

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Transmittal New Application

OUTGOING

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Misc. Office Action

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Signed 1449

7/11/02 892 1
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Abandonment

_____ APDEC _____
Board of Appeals Decision

_____ APEA _____
Examiner Answer

_____ CTAV _____
Count Advisory Action

_____ CTEQ _____
Count Ex parte Quayle

_____ CTFR _____
Count Final Rejection

_____ CTNF _____
Count Non-Final

_____ CTRS _____
Count Restriction

_____ EXIN _____
Examiner Interview

_____ M903 _____
DO/EO Acceptance

_____ M905 _____
DO/EO Missing Requirement

_____ NFDR _____
Formal Drawing Required

_____ NOA _____
Notice of Allowance

_____ PETDEC _____
Petition Decision

INCOMING

_____ AP.B _____
Appeal Brief

_____ C.AD _____
Change of Address

_____ N/AP _____
Notice of Appeal

_____ PA.. _____
Change in Power of Attorney

_____ REM _____
Applicant Remarks in Amendment

_____ XT/ _____
Extension of Time filed separate

Internal

_____ SRNT _____
Examiner Search Notes

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PTO Prepared Complete Claim Set

_____ ECBOX _____
Evidence Copy Box Identification

_____ WCLM _____
Claim Worksheet

_____ WFEE _____
Fee Worksheet

File Wrapper

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File Wrapper Claim

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File Wrapper Issue Information

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File Wrapper Search Info

Notice of References Cited	Application/Control No. 09/725,019	Applicant(s)/Patent Under Reexamination THOMPSON ET AL.	
	Examiner Stuart F. Baum	Art Unit 1638	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-5,763,742	06-1998	Morrison et al	800/200
	B	US-5,767,364	06-1998	de Silva et al	800/205
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Fourgoux-Nicol et al (1999, Plant Molecular Biology 40 :857-872)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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Foreign Priority Papers

_____ IDS _____
IDS Including 1449

7/11/02 NPL ✓
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Petition Decision

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892

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Abandonment

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Board of Appeals Decision

_____ APEA _____
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File Wrapper Search Info

REV 7/02/03

Plant Molecular Biology **40:** 857–872, 1999.

Isolation of rapeseed genes expressed early and specifically during development of the male gametophyte

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Key words: Arabidopsis, gene-specific expression, GUS staining, *in situ* hybridization, microspore, rapeseed

Abstract

A cDNA subtraction and differential hybridization strategy was used to isolate cDNAs expressed early during male gametophyte development in the important crop species *Brassica napus*. Three cDNAs, corresponding to genes highly and specifically expressed at the tetrad and microspore stages, are presented here. The analysis of one of them, named *BnM3.4*, by *in situ* hybridization, showed that it is expressed specifically and at a high level in the rapeseed microspore. The specificity in its profile of expression is most likely transcriptionally controlled as a similar pattern of expression was also observed in *Arabidopsis thaliana* plants transformed by the *BnM3.4* promoter fused to the reporter GUS-coding sequence. The putative *BnM3.4* promoter contains three dispersed copies of a motif described previously in the promoters of several genes expressed in the male gametophyte. The *BnM3.4* gene encodes a predicted novel proline-rich protein of 23.4 kDa which may interact with cytoskeletal components or have a structural role in the cell wall.

Introduction

Pollen development in angiosperm plants occurs in a specialized floral organ, the anther. It starts by a series of mitotic divisions of archeosporial cells from the primary sporogenous layer leading to the formation of meiocytes. Each meiocyte undergoes meiosis to generate a tetrad of four haploid microspores surrounded by a callose wall, whose digestion then liberates them into the anther locule. A complex extracellular matrix composed of intine and exine is built around the male gametophyte after microspore differentiation. An asymmetric cell division of each microspore produces the bicellular pollen grain in which the large vegetative cell encloses the smaller generative cell. This latter cell has a condensed nucleus and a reduced cytoplasm. In the majority of plant species, the second mitotic division of the generative cell occurs after pollination within the growing pollen tube producing two sperm cells. In some genera such as *Brassica* this second pollen mitosis occurs earlier during pollen maturation. The pollen grain is hence released from the anther locule in a tricellular form. This developmental programme is necessary to prepare the pollen grain for efficient interaction with the stigma, for rapid germination and pollen tube growth, and for successful delivery of sperm cells to the ovules (Bedinger *et al.*, 1994).

Despite the wealth of descriptive studies on the structural and physiological aspects of pollen formation, our understanding of the molecular events remains rather limited. Our main goal is to extend our knowledge on microspore differentiation and development through the identification and characterization of novel genes specifically expressed during early stages of microgametogenesis. Such genes may also provide alternative methods for controlling male fertility in economically important plants, such as in our case *Brassica napus*. To identify genes that might be

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF136223 (*BnM3.4*).

implicated in the developmental process, differential screening of cDNA libraries constructed from whole anthers has been the most commonly used approach. A number of genes have been shown to be expressed in developing pollen, as well as in the tapetum or other sporophytic tissues of the anther (Ursin *et al.*, 1989; Koltunow *et al.*, 1990; Nacken *et al.*, 1991; Theerakulpisut *et al.*, 1991; Paul *et al.*, 1992; Aguirre and Smith, 1993; Robert *et al.*, 1993; Bucciaglia and Smith, 1994; Ross and Murphy, 1996). Some pollen-specific genes expressed after the first mitotic division and presumably playing a role in pollen development, maturation, germination or pollen tube growth have also been described (Hanson *et al.*, 1989; Brown and Crouch, 1990; Shen and Hsu, 1992; Weterings *et al.*, 1992; Brander and Kuhlemeier, 1995; Stanchev *et al.*, 1996; Yu *et al.*, 1998). Finally, so far, a single tobacco gene has been identified that shows a microspore-specific expression (Oldenhof *et al.*, 1996),

We report the isolation of cDNAs from *B. napus* that are expressed during early pollen development. During this work, we developed a novel combined approach by differential screening of a microspore cDNA library with a subtracted probe enriched for microspore-specific sequences. We describe here in detail the characterization of the structure and expression of the *BnM3.4* gene corresponding to the microspore-specific cDNA M3.

Materials and methods

Plant material

Plants of *B. napus* L. cv. Brutor were grown in an open field or under standard greenhouse conditions and were used for RNA and DNA isolation. *Arabidopsis thaliana* (Wassilevskija ecotype) plants used for transformation (Bechtold *et al.*, 1993) were grown in a greenhouse under standard conditions. Transgenic *A. thaliana* T_1 seedlings were selected in a greenhouse on sand, sub-irrigated with water containing Basta herbicide (7.5 mg/l phosphinothricine). Two months later, T_2 seeds were harvested individually. *In vitro* culture of seedlings for segregation analysis was done in a culture chamber on *A. thaliana* medium (Estelle and Sommerville, 1987) containing 5 mg/l of phosphinothricine as selective agent.

RNA isolation and poly(A)$^+$ RNA purification

Several grams of rapeseed flower buds from 0.2 mm to more than 4 mm in length were harvested in 4 different classes according to bud length. Bud length was measured from the base to the tip of the outermost sepal. Male gametophytes from graded floral buds were isolated and purified as previously described (Albani *et al.*, 1990) with a few modifications. After disruption of the buds in a blender with a solution of 10% sucrose (pH 7), the resulting suspension was filtered through 80 μm nylon mesh. Only the suspension containing microspores (from 2 to 3 mm buds) was filtered through 45 μm nylon mesh. After two washes, as described by Albani *et al.* (1990), the pellet was frozen in liquid nitrogen and stored at −80 °C if not immediately used for RNA extraction. The purified male gametophytes from the different classes of bud were disrupted by 3 cycles of pressurization (at 110 bar) depressurization in a mini-bomb (Bioblock Scientific, Illkirch, France) in 50% RNA extraction buffer and 50% phenol before performing RNA extraction as previously reported (Dean *et al.*, 1985).

Poly(A)$^+$ RNAs were purified from total RNA using the mRNA purification kit from Pharmacia Biotech.

Total RNA was extracted from young rapeseed seedlings, total fertile and male-sterile buds, pistils, sepals and petals, roots, leaves and stems as described above.

cDNA library construction and cDNA subtraction

The procedure used for the isolation of organ-specific cDNA was based on an unpublished protocol from S. Lok and D.C. Baulcombe (Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK). It is the result of several reports on ligation-mediated PCR (Mueller and Wold, 1989), direct incorporation of biotin nucleotide during PCR (Lo *et al.*, 1988) and differential removal of biotinylated DNA by a streptavidin/phenol extraction procedure (Sive and St John, 1988; Wang and Brown, 1991) and was modified as follows.

The blunted 'tracer' cDNA (T) and the 'driver' cDNA (D) were synthesized from 3 μg of poly(A)$^+$ RNA from microspores and male-sterile buds (Ogu-INRA) (Gourret *et al.*, 1992), respectively, using a kit from Pharmacia Biotech. An aliquot of the tracer cDNA was ligated to the *Eco*RI/*Not*I adaptor according to the kit's instructions and inserted into the